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***In vivo* and *in vitro* analyses of α -galactosylceramide uptake by conventional dendritic
cell subsets using its fluorescence-labeled derivative**

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Abbreviations:

α GC, α -galactosylceramide; cDCs, conventional dendritic cells; Cy5- α GC, Cy5-conjugated

α GC; iNKT cells, invariant natural killer T cells

Abstract

Conventional dendritic cells (cDCs) present α -galactosylceramide (α GC) to invariant natural killer T (iNKT) cells through CD1d. Among cDC subsets, CD8⁺ DCs efficiently induce IFN- γ production in iNKT cells. Using fluorescence-labeled α GC, we showed that CD8⁺ DCs incorporated larger amounts of α GC and kept it intact longer than CD8⁻ DCs. Histological analyses revealed that Langerin⁺CD8⁺ DCs in the splenic marginal zone, which was the unique equipment to capture blood-borne antigens, preferably incorporated α GC, and the depletion of Langerin⁺ cells decreased IFN- γ and IL-12 production in response to α GC. Furthermore, splenic Langerin⁺CD8⁺ DCs expressed more membrane-bound CXCL16, which possibly anchored iNKT cells in the marginal zone, than CD8⁻ DCs. Collectively, it is suggested that the cellular properties and localization of CD8⁺ DCs are important for stimulation of iNKT cells by α GC.

Keywords: α -galactosylceramide; Dendritic cells; IFN- γ ; iNKT cells; Subsets

1. Introduction

Dendritic cells (DCs) are antigen-presenting cells (APCs) that exist widely in the body and survey pathogen invasions [1]. Upon recognition of pathogens, DCs present peptide antigens via major histocompatibility complex (MHC) molecules to the T cells. DCs also present lipid antigen via nonclassical MHC CD1 to natural killer T cells (NKT cells) [2], inducing robust cytokine production.

DCs are primarily divided into two populations: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [3]. cDCs are further subdivided into two groups, CD8⁺ and CD8⁻ DCs, by the expression of CD8 α [4]. NKT cells are divided into two subsets by the pattern of T cell antigen receptor (TCR) expression. Type I NKT cells express invariant TCR, so they are designated as invariant NKT (iNKT) cells [5]. iNKT cells recognize marine sponge α -galactosylceramide (α GC) on the CD1d molecule of cDCs, inducing antitumor activity via the robust production of IFN- γ . During interaction with cDCs, iNKT cells express CD40 ligand (CD40L), which stimulates cDCs via their CD40, subsequently inducing IL-12 production from CD8⁺ DCs. In turn, IL-12 stimulates iNKT cells, resulting in the strong production of IFN- γ [6,7] Furthermore, using Langerin-eGFP-diphtheria toxin receptor (DTR) mice, depletion of Langerin⁺ cDCs decreased IL-12 and IFN- γ production after stimulation with injection of α GC [8], suggesting that Langerin⁺CD8⁺ DCs are involved in IFN- γ production of iNKT cells *in vivo*. However, it is unclear why CD8⁺ DCs induce higher IFN- γ production of iNKT cells than CD8⁻ DCs.

After *i.v.* injection, antigens in the blood are captured by the splenic marginal zone (MZ) cells, causing a rapid immune response [9]. MZ consists of SIGNR1/ER-TR9⁺ macrophages, MZB cells, and cDCs, forming special equipment to sense/capture antigens in the blood flowing into the sinus between the MZ and marginal CD169/SER4⁺ metallophil zone. Interestingly, Langerin⁺CD8⁺ DCs were observed in the MZ, and iNKT cells migrated to the MZ after injection of α GC [10].

Here, we compared α GC uptake and degradation between CD8⁻ and CD8⁺ DC subsets using Cy5-conjugated α GC (Cy5- α GC). We also performed histological analyses to observe the uptake of Cy5- α GC in the spleen. Our results suggest that both cellular properties and localization of CD8⁺ DCs enable efficient stimulation of iNKT cells in response to α GC.

2. Materials and Methods

2.1. Mice

C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan), J α 18 knock out (KO) mice (B6 background) were provided from Dr. Taniguchi (RIKEN RCAI, Yokohama, Japan), and Langerin-diphtheria toxin receptor (DTR) mice (B6 background) were provided by Dr. Kabashima (Kyoto University, Kyoto, Japan). All mice were housed under specific pathogen-free conditions and used at 7–12 wk of age. All experiments were conducted according to our institutional guidelines.

2.2. Reagents and antibodies

α GC (Funakoshi Co., Ltd., Tokyo, Japan) was dissolved at 200 μ g/ml in 0.5% Tween-20 in PBS. Cy5- α GC was provided from Dr. Watarai (The University of Tokyo, Tokyo, Japan). Diphtheria toxin (DT) and 30% bovine serum albumin (BSA) solution were purchased from Sigma-Aldrich (St Louis, MO). 7-Aminoactinomycin D (7-AAD) was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). RPMI 1640 was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan).

Fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (N418), phycoerythrin (PE)-conjugated anti-CD8 α (53-6.7) and anti-CD11c mAbs were obtained from eBioscience (San Diego, CA, USA). FITC-conjugated anti-CD31 (MEC13.3) and allophycocyanin (APC)-conjugated anti-CD8 α (53-6.7) mAb were obtained from BD Biosciences (San Diego,

CA, USA). Anti-Langerin (L31) and anti-CXCL16 (12-18) [11] mAbs were provided by Dr. Steinman (Rockefeller University, New York, USA) and Dr. Yonehara (Kyoto University, Kyoto, Japan), respectively. Anti-CXCL16 mAb was biotinylated in our laboratory. Anti-SER4 mAb was purified from culture supernatant of hybridoma and biotinylated in our laboratory. Streptavidin-PE was purchased from BD Biosciences. Cy3-conjugated anti-rat IgG was purchased from Jackson ImmunoResearch Europe Ltd. (Cambridgeshire, UK).

Hybridomas producing anti-CD16/32 mAb (2.4G2) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and culture supernatants were produced in our laboratory.

MACS microbeads conjugated with anti-mouse CD11c and FITC were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

2.3. Cell preparation

cDCs were prepared as follows: spleens were treated with 400 U/ml collagenase D (Roche, Mannheim, Germany) for 45 min at 37°C, and suspended. Subsequently, low density cells were obtained by centrifugation at $800 \times g$ for 30 min using 30% BSA. CD11c⁺ cells were purified from the cells using anti-CD11c microbeads following the manufacturer's protocol. To check depletion of CD8⁺ DCs by DT treatment, whole splenocytes after the collagenase digestion were subjected to flow cytometry. For measurement of CXCL16 on the cell surface, the spleen was dispersed in 5 mM EDTA in PBS and whole splenocytes were

analyzed using flow cytometry.

2.4. Flow cytometry of cell populations and Cy5- α GC uptake

Fc receptors on cells were blocked with anti-CD16/32 mAb in staining buffer for 30 min on ice, and stained for 30 min on ice with mAbs. Data were collected on FACSCalibur and were analyzed with FlowJo (TreeStar, Ashland, OR). Net MFI was obtained by subtraction of the isotype mAb MFI from that of specific mAb.

For the *in vivo* assay of Cy5- α GC uptake, J α 18 KO mice were injected *i.v.* with 5 μ g Cy5- α GC in 200 μ L PBS. At various time points, splenocytes were stained with anti-CD11c-FITC, anti-CD8-PE, and Cy5- α GC incorporation of cDCs was measured by flow cytometry. The net uptake of Cy5- α GC was calculated by subtracting MFI of Cy5 fluorescence of untreated mouse cDCs from that of treated mouse cDCs.

For the *in vitro* assay of Cy5- α GC uptake, cDCs stained with anti-CD11c-FITC and anti-CD8-PE were cultured in the presence of graded doses of Cy5- α GC for 1 h, or of 100 ng/mL α GC for various time points. After incubation, Cy5-fluorescence intensity was measured gating on CD11c⁺CD8⁺ and CD11c⁺CD8⁻ by flow cytometry. Autofluorescence intensity and background binding of Cy5- α GC by cDCs were calculated by subtraction of fluorescence intensity (MFI) of cDCs cultured with Cy5- α GC at 4°C from that of cDCs cultured at 37°C. In pulse and chase experiments, net MFI was calculated by subtraction of autofluorescence intensity of cDCs from MFI of cDCs cultured with Cy5- α GC at 37°C.

The % reduction was calculated using the following formula: [MFI of Cy5- α GC at the time point / that at the initial time point \times 100]. In some cases, NH_4Cl (20 mM) was added in the culture.

2.5. Cytokine assay

To observe cytokines in sera, mice were injected *i.v.* with 5 μg α GC. After 2 or 4 h, blood was collected. After clotting, IL-12p70, IL-4, and IFN- γ in the sera were assessed by a cytometric bead array (CBA, BD Bioscience). In some cases, Langerin-DTR mice or B6 mice were treated two times, 48 h apart, with 350 ng of DT or vehicle by *i.p.* injection to deplete Langerin⁺ cells.

2.6. Immunostaining

Spleens were frozen in Tissue-Tek OCT-compound (SAKURA SEIKI Co., Ltd, Tokyo, Japan) at -20 °C. Cryostat sections (10 μm in thickness) were dried, fixed in acetone for 10 min and then dried again. After Fc blocking with anti-CD16/32 mAb, sections were stained for 1 h with antibodies in staining buffer (1% FCS, 0.02% NaN_3 , 5 mM EDTA in PBS). Images were obtained with a fluorescence microscope BX51 (OLYMPUS, Tokyo, Japan). In some cases, mice were pulsed *i.v.* with Cy5- α GC, and spleen sections were prepared after 15 min. To observe the distribution of Langerin⁺ DCs in the spleen, BALB/c mice were used, because of the low expression of Langerin in B6 mice.

2.7. Statistical analysis

Data are expressed as the mean \pm SD of triplicate cultures or three mice in each group.

Statistical significances were determined by the Student's-*t* test. Differences were considered significant for *p* values < 0.05. All experiments were performed at least two times and representative results are shown.

3. Results and Discussion

3.1. $CD8^+$ DCs effectively uptake more Cy5- α GC than $CD8^-$ DCs *in vivo*.

We first confirmed previous results showing higher presentations of α GC and induction of IFN- γ production from iNKT cells by $CD8^+$ DCs in comparison with $CD8^-$ DCs. After *i.v.* injection, α GC presentation on cDC subsets was measured at various time points (Supplementary Fig. 1A) using mAb specific to the α GC/CD1d complex [12], indicating that $CD8^+$ DCs expressed larger amounts of the α GC/CD1d complex than $CD8^-$ DCs as reported previously [13]. Consistently, $CD8^+$ DCs upon loading with α GC *in vivo* induced higher IFN- γ after co-culture with naïve iNKT cells (Supplementary Fig. 1B). For IFN- γ production, IL-12 produced by $CD8^+$ DCs was indispensable (Supplementary Fig. 1C, D). To acquire the IFN- γ inducing activity via IL-12 production, cDCs require interaction *in vivo* with iNKT cells during α GC presentation (Supplementary Fig. 1E) as reported previously [6].

Then, we compared α GC uptake using its fluorescent derivative between $CD8^+$ and $CD8^-$ DCs *in vivo*. For precise comparisons, we used $J\alpha 18$ KO mice that lacked iNKT cells [14], as iNKT cells possibly killed mature cDCs that presented α GC. The mice were injected with Cy5- α GC, and fluorescence of cDC subsets was checked at various time points (Fig. 1), indicating that $CD8^+$ DCs incorporated larger amounts of Cy5- α GC than $CD8^-$ DCs at all time points. This result suggests that $CD8^+$ DCs uptake α GC more efficiently than $CD8^-$ DCs *in vivo*, leading to the robust induction of IFN- γ from iNKT cells.

3.2. $CD8^+$ DCs phagocytose more Cy5- α GC and keep it intact longer than $CD8^-$ DCs *in vitro*.

We then observed the uptake of Cy5- α GC in $CD8^+$ and $CD8^-$ DCs *in vitro*. Purified $CD8^+$ and $CD8^-$ DCs were co-cultured *in vitro* with Cy5- α GC to observe its incorporation. Consistent with the *in vivo* results at all concentrations of Cy5- α GC (Fig. 2A) and incubation time points (Fig. 2B) tested, $CD8^+$ DCs showed higher Cy5 fluorescence intensity than $CD8^-$ DCs. Besides, like protein antigens, α GC is loaded onto CD1d in endosomes/lysosomes [2], and endosome pH in $CD8^+$ and $CD8^-$ DCs is about 7 and 6, respectively [15], suggesting that stability of α GC in endosomes/lysosomes is different between them. To clarify this, we treated cDCs with NH_4Cl to inhibit endosome/lysosome acidification. When NH_4Cl was added in culture, $CD8^+$ DCs did not change the intensity of Cy5 fluoresce (Fig. 2C). However, $CD8^-$ DCs increased Cy5 fluorescence, but it was still lower than that of $CD8^+$ DCs. We determined that Cy5 fluorescence was not affected from pH 7 to 4.5 (data not shown). These results suggest that $CD8^+$ DCs take up more Cy5- α GC than $CD8^-$ DCs, and that Cy5- α GC is degraded by the low pH of endosomes in $CD8^-$ DCs. To further clarify this, cDCs were pulsed with Cy5- α GC for 30 min, and a change of fluorescence intensity was observed (Fig. 2D). The results indicated that $CD8^-$ DCs lost Cy5 fluorescence faster than $CD8^+$ DCs. We then confirmed the presentation of the α GC/CD1d complex on the DCs using flow cytometry. The results indicated that $CD8^+$ DCs presented larger amounts of α GC/CD1d complex than $CD8^-$ DCs at the concentrations we tested (Figs. 2E, F).

When endosomal pH in DCs is up-regulated, the loading activity of α GC by CD1d decreases [2,16], suggesting lower efficiency of α GC presentation of CD8⁺ DCs than that of CD8⁻ DCs. However, CD8⁺ DCs presented larger amounts of α GC than CD8⁻ DCs *in vivo* and *in vitro*. Therefore, CD8⁺ DCs seem to accumulate higher amounts of α GC than CD8⁻ DCs, overcoming their low efficiency of α GC loading in high endosomal pH. Collectively, CD8⁺ DCs effectively incorporate α GC and keep it intact, leading to the higher α GC/CD1d presentation compared to CD8⁻ DCs.

3.3. *Langerin⁺CD8⁺ DCs in MZ capture α GC, and are crucial for cytokine production from iNKT cells.*

Langerin is one of the specific markers of splenic CD8⁺ DCs (Fig. 3A). Immunostaining with anti-Langerin mAb revealed that CD8⁺ DCs were primarily localized at the MZ, outer layer of SER4⁺ marginal metallophil, and bridging area (Fig. 3B). After *i.v.* injection, Cy5- α GC was observed in CD11c positive cells of MZ (Fig. 3C) and CD31⁺ endothelial cells of blood vessels (Fig. 3D). A weak Cy5 signal was also detected in the B cell area (Fig. 3C). Including the results above (Supplementary Fig. 1 and Fig. 1), these findings suggest that Langerin⁺CD8⁺ DCs are strategically located in MZ, especially adapted for sensing blood-borne antigens, and preferentially capture α GC in the spleen.

To define the relevance of capturing and presentation of α GC by the Langerin⁺CD8⁺ DCs, Langerin-DTR mice were treated with DT and injected *i.v.* with α GC. As shown in Fig.

3E, DT treatment effectively decreased CD8⁺ DCs in Langerin-DTR mice. In the control (PBS) treatment, Langerin-DTR mice produced comparative level of IL-12, but lower levels of IFN- γ and IL-4, compared to WT mice after α GC administration (Fig. 3F). The reason for such low levels of IFN- γ and IL-4 is not clear. Nevertheless, in Langerin-DTR mice, DT treatment significantly decreased amounts of IFN- γ and IL-12, not IL-4, as previously reported by Farrand et al. [8]. Therefore, it is suggested that Langerin⁺CD8⁺ DCs in MZ capture α GC and lead to *in vivo* IFN- γ production by iNKT cells.

3.4. CD8⁺ DCs express larger amounts of membrane-bound CXCL16 than CD8⁻ DCs.

CXCL16 is a ligand of CXCR6 expressed in iNKT cells [17]. Although no difference of iNKT cell numbers was observed between WT and CXCR6-lacking mouse spleens, after treatment with α GC, IFN- γ -producing iNKT cells decreased significantly in the deficient mice [18]. It has also been suggested that membrane-bound CXCL16 on DCs mediates interactions with CXCR6 expressed on iNKT cells, leading to their adhesion/anchoring and full activation of iNKT cells [11,19]. However, the expression of membrane-bound CXCL16 on cDC subsets is unknown. Before stimulation with α GC, CD8⁺ DCs expressed higher amounts of membrane-bound CXCL16 than CD8⁻ DCs (Fig. 4). Four hours after α GC injection, the expression by CD8⁺ DCs did not change. CD8⁻ DCs expressed small amounts of membrane-bound CXCL16 regardless of α GC injection. King et al. reported that iNKT cells were widely localized in the spleen in the resting state and subsequently migrate into MZ after

treatment with α GC [10]. Therefore, it is possible that the membrane-bound CXCL16 adherers/anchors facilitated the migration of iNKT cells to CD8⁺ DCs in MZ after α GC injection. The other possibility is that anchoring of small numbers of naïve iNKT cells in MZ at a steady state leads to the effective recognition of α GC presented by CD8⁺ DCs during the first step of the immune response. As mentioned above (Supplementary Fig. 1E), iNKT cells are indispensable for acquiring iNKT cell activation by CD8⁺ DCs *in vivo*. Therefore, in both cases, it seems that efficient adhesion of CD8⁺ DCs with iNKT cells via CXCL16–CXCR6 interaction is involved in the activation of CD8⁺ DCs and subsequent IFN- γ production of iNKT cells.

4. Conclusion

In cDC subsets, CD8⁺ DCs uptake α GC effectively compared with CD8[−] DCs *in vivo* and *in vitro*. In addition, α GC is more stable in CD8⁺ DCs than in CD8[−] DCs. It is possible that these two properties of CD8⁺ DCs result in their efficient accumulation and presentation of α GC. Furthermore, CD8⁺ DCs strategically localize in the MZ, which is specialized in capturing blood-borne antigens, and preferentially uptake α GC injected *i.v.* Furthermore, CD8⁺ DCs in the spleen express higher levels of membrane-bound CXCL16 than CD8[−] DCs, possibly enhancing activation of iNKT cells. These cellular properties and localization of CD8⁺ DCs seem to enable the robust induction of IFN- γ from iNKT cells *in vivo*.

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Author contributions

MU, TI, and KI designed the study. MU mainly performed experiments with the help of TI and MK. HW provided Cy5- α GC. MU and KT wrote the manuscript.

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Figure legends

Fig. 1. CD8⁺ DCs took up more α GC than CD8⁻ DCs *in vivo*.

J α 18 KO mice (n = 4) were injected *i.v.* with 5 μ g of Cy5- α GC. At the time points indicated, Cy5 fluorescence of splenic CD8⁺ and CD8⁻ DCs was measured by flow cytometry.

Fig. 2. CD8⁺ DCs were superior in α GC-uptake and its retention to CD8⁻ DCs *in vitro*.

(A) Purified DCs were cultured with the indicated doses of Cy5- α GC. After 1 h, Cy5 fluorescence of CD8⁺ and CD8⁻ DCs was measured by flow cytometry. (B) Purified DCs were cultured with 100 ng/mL Cy5- α GC for the time points indicated, and Cy5 fluorescence was analyzed. (C) NH₄Cl was added to the culture, and Cy5 fluorescence was analyzed as in (B). (D) After culture with 100 ng/mL Cy5- α GC for 30 min, purified cDCs were cultured, and change of MFI of Cy5 fluorescence at the various time points was analyzed. (E) Low-density spleen cells were cultured with or without 100 ng/mL non-labeled- α GC for 4 h, followed by staining with anti-CD11c-FITC, anti-CD8-APC, PE-anti- α GC/CD1d complex, and 7-AAD. 7-AAD⁻ live cells were analyzed. Solid lines and shadows indicate intensity of anti- α GC/CD1d complex and isotype control mAb, respectively. (F) Low-density cells were cultured with graded doses of α GC for 4 h, and level of α GC/CD1d complex was analyzed as in (E). **p* < 0.01 by Student's *t*-test.

Fig. 3. DC8⁺ DCs in the splenic marginal zone took up Cy5- α GC.

(A) Expression of Langerin on cDC subsets from BALB/c naïve mice was analyzed. Solid lines and shadows indicate staining of anti-Langerin and isotype control mAb, respectively. (B) BALB/c mouse spleen section was stained for CD11c (green), Langerin (red), and SER4 (white). T: T area; B: B area; MZ: marginal zone; CA: central arteriole. Bar indicates 100 μ m. (C, D) After 15 min of injection of Cy5- α GC (red), the section was stained for CD11c (green) and SER4 (white) (C), or CD31 (white) (D). Bar indicates 100 μ m. (E) CD8⁺ DCs in spleen of WT and Langerin-DTR mice (B6 background) were analyzed after control (PBS) or DT treatment by flow cytometry. Numbers indicate percentages of CD8⁺ DCs in 7AAD⁻ splenocytes. (n = 3) (F) W

(B) T mice (n = 6) and Langerin-DTR mice (n = 7) treated with PBS or DT were injected *i.v.* with 5 μ g of α GC and sera were collected at 2 h (IL-4) and 4 h (IFN- γ , IL-12). Cytokines were measured by cytometric bead array (CBA). **p* < 0.01 by Student's *t*-test. N. S.: not significant.

Fig. 4. CD8⁺ DCs expressed larger amounts of membrane-bound CXCL16 than CD8⁻ DCs.

Expression of CXCL16 on cDC subsets from B6 mice was analyzed 4 h after *i.v.* injection with 5 μ g of α GC or vehicle by flow cytometry. Solid lines and shadows indicate staining of anti-CXCL16 and isotype control mAb, respectively (left panels). The results from three mice are depicted (right panel). N. S.: not significant.

Biographies of authors

Maki Ushida

She is a doctoral student of Kyoto University. After undergraduate course of Kyoto Pharmaceutical University, she entered Graduate School of Biostudies, Kyoto University at 2008. She is studying about the mechanism of lipid antigen presentation in dendritic cells, professional antigen presenting cells.

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Hiroshi Watarai

After graduation of Graduate School of Agricultural and Life Sciences at the University of Tokyo, Watarai became involved in the research and development of recombinant proteins and monoclonal antibodies as next generation drugs at Pharmaceutical Division of Kirin Brewery Co. Ltd. (the current Kyowa Hakko Kirin). After conducting research at RIKEN and JST, Watarai reached his current position in 2014.

Kazuhiko Takahara

Lecturer of Kyoto University. After a master's program of Hiroshima University, he worked as a researcher in a private company, and visited Osaka University (Japan) and National Cancer Center (Japan) for studying about gene mapping and functions of collagen, respectively. He obtained Ph.D. degree from Hiroshima University at 1992. After quitting the company at 1993, he visited University of Wisconsin-Madison (USA) as a post doctoral fellow. From 1997, he is a faculty member of Kyoto University. He was awarded Outstanding Merit Award from International Immunology in 2004 and 2008.

Kayo Inaba

Prof. Kayo Inaba is currently serving as Exe. Vice-President of Kyoto University. Prof. Inaba is working in Graduate School of Biostudies of Kyoto University and one of the world

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leading scientist of dendritic cell research, and her work has been published and cited in many international journals. She took her Ph.D. from Kyoto University in 1978. She received many awards and honors, such as L'Oreal UNESCO Award for Women in Science, Kyoto University Shishi Prize, and Akebono Prize. She is also serving as board member of the Japanese Society for Immunology, Vice President of the Japanese Dendritic Cell Society, etc.

Highlights:

- 1) We saw the fate of fluorescent α -galactosylceramide (Cy5- α GC) *in vivo* and *in vitro*.
- 2) CD8⁺ dendritic cells (DCs) captured more α GC than CD8⁻ DCs *in vivo* and *in vitro*.
- 3) Cy5- α GC was more stable in CD8⁺ DCs than in CD8⁻ DCs.
- 4) Langerin⁺CD8⁺ DCs in the splenic marginal zone incorporated Cy5- α GC.
- 5) Splenic CD8⁺ DCs expressed more CXCL16, which might anchor iNKT cells, than CD8⁻ DCs.

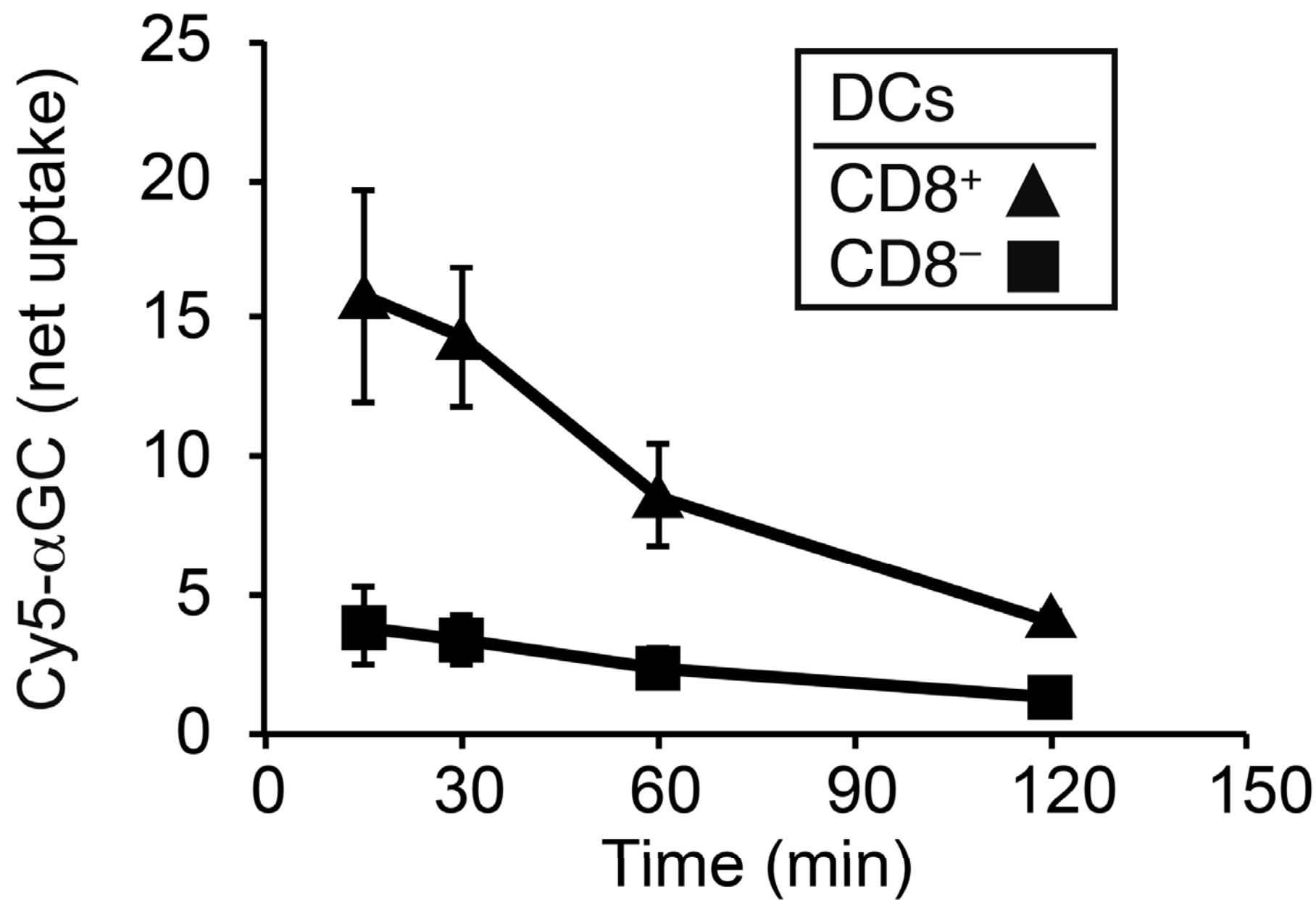


Fig. 1

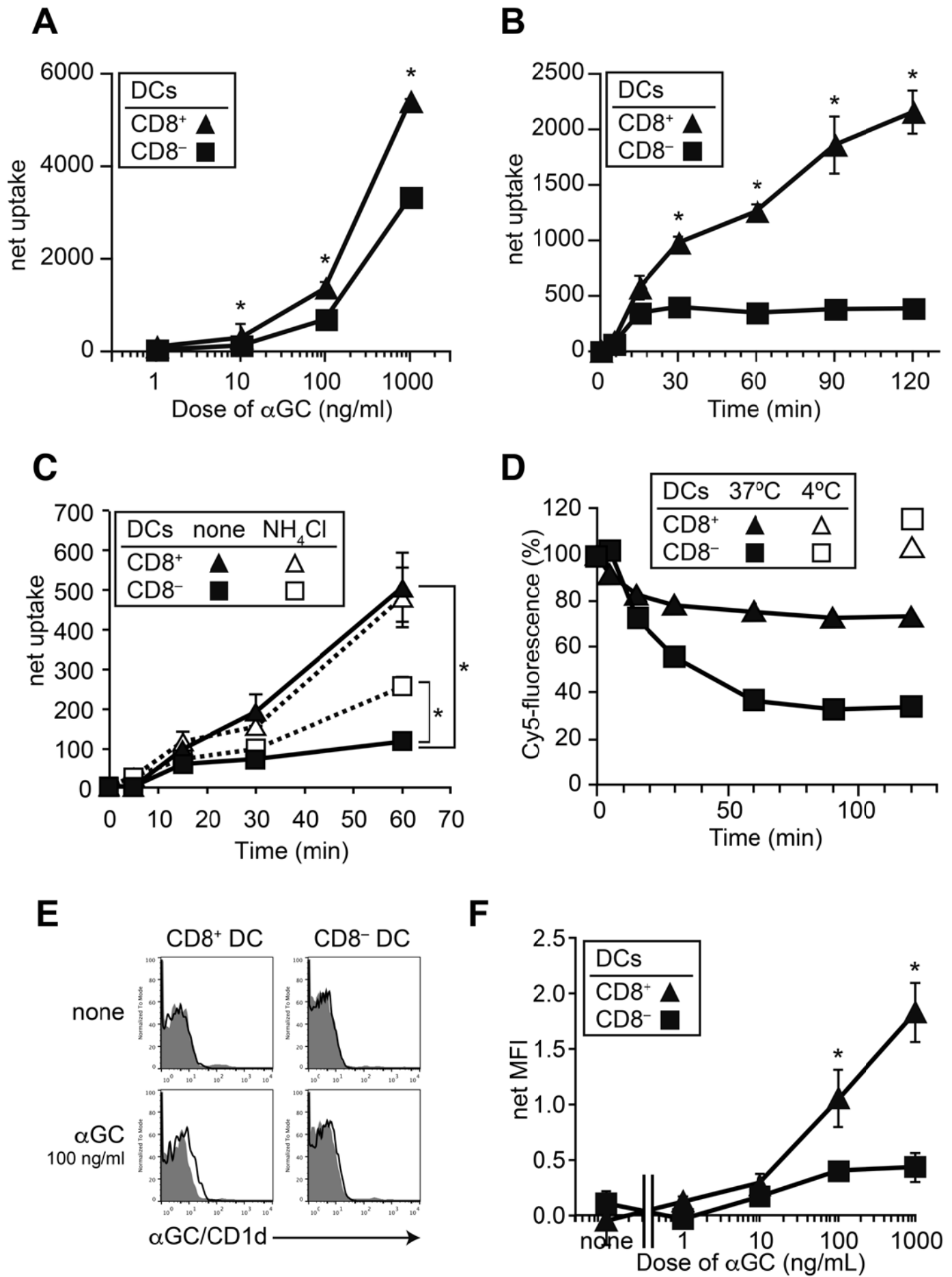


Fig. 2

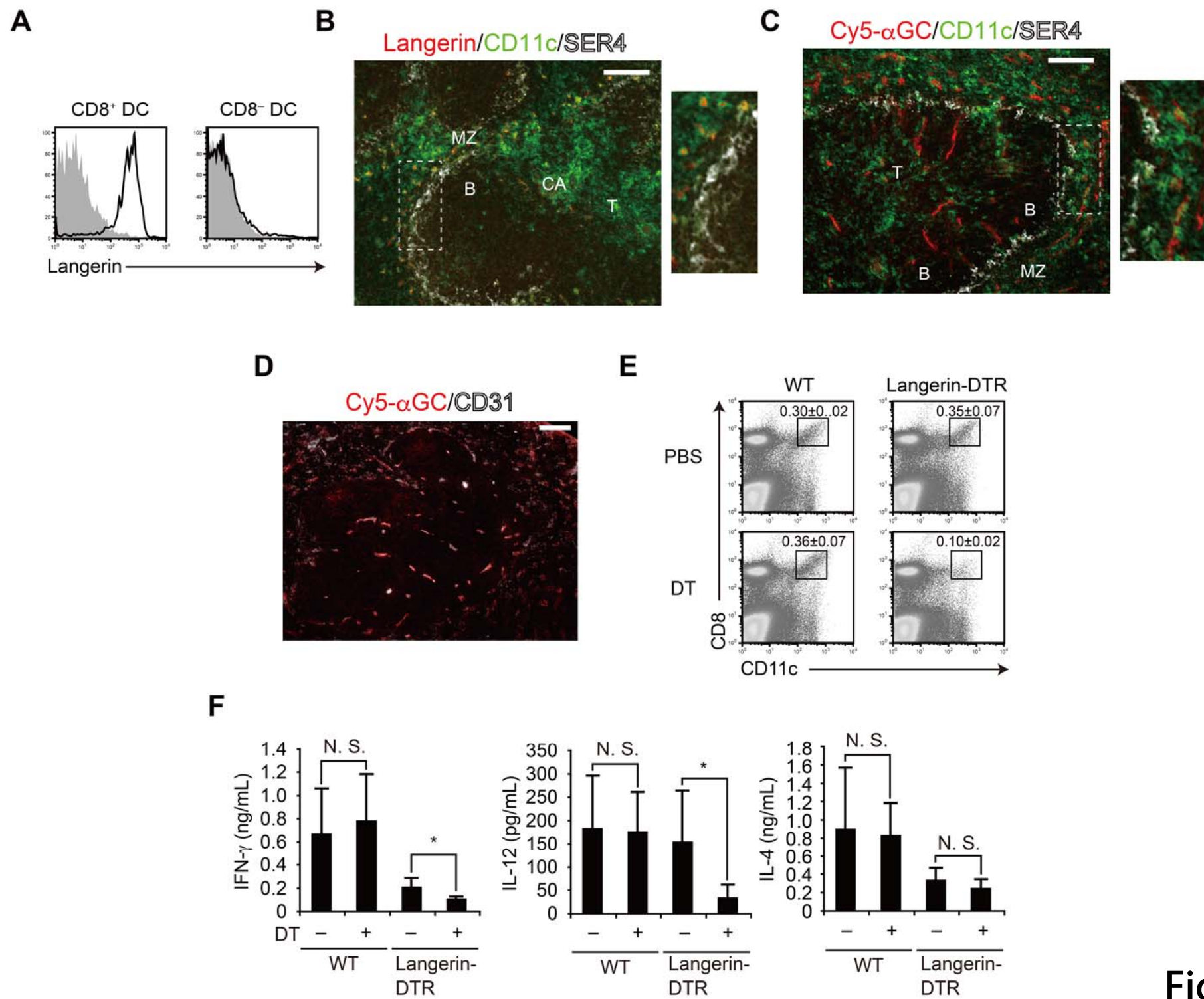


Fig. 3

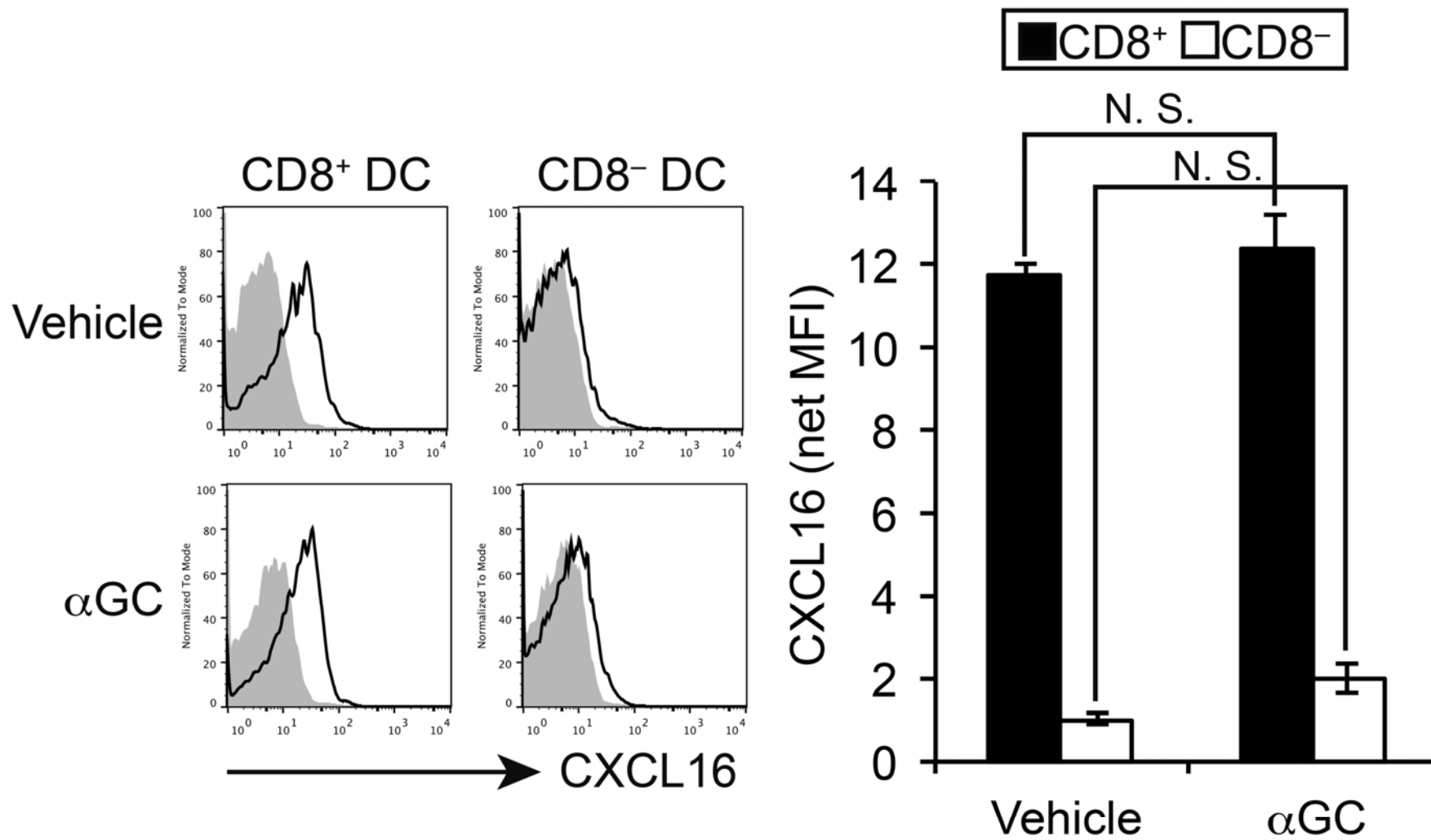


Fig. 4

Supplementary information

1. Supplementary Materials and Methods

1.1. Reagents and antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (N418), phycoerythrin (PE)-conjugated anti-CD8 α (53-6.7), and anti- α GC/CD1d (L363) mAb were obtained from eBioscience (San Diego, CA, USA). FITC-conjugated anti-CD19 (1D3), PE-conjugated anti-mouse IgG₁ (A85-1), and allophycocyanin (APC)-conjugated anti-CD8 α (53-6.7) mAb were obtained from BD Biosciences (San Diego, CA, USA). Dimer X (mouse CD1d-Ig) was purchased from BD Biosciences. Neutralizing anti-IL-12 mAb was purchased from Biosource international (Camarillo, CA, USA).

Hybridomas producing anti-MHC class II (M5/14.15.2), anti-CD8 α (53-6.72), anti-CD62L (Mel-14), anti-CD11b (M1/70), and anti-B220 (RA3-6B2.1) were purchased from ATCC, and culture supernatants were produced in our laboratory.

MACS microbeads conjugated with anti-mouse CD11c was from Miltenyi Biotec GmbH and Dynabeads conjugated with sheep anti-rat IgG from Invitrogen Life Technologies.

1.2. Cell preparation, stimulation, and culture

Purified cDCs subsets and iNKT cells were prepared as described previously [1]. Briefly, for cDC subsets preparation, purified CD11c⁺ cells using anti-CD11c microbeads were stained with anti-CD11c-FITC and anti-CD8-PE, and sorted into CD8⁺ and CD8⁻ DC with FACS

VantageSE (BD Biosciences).

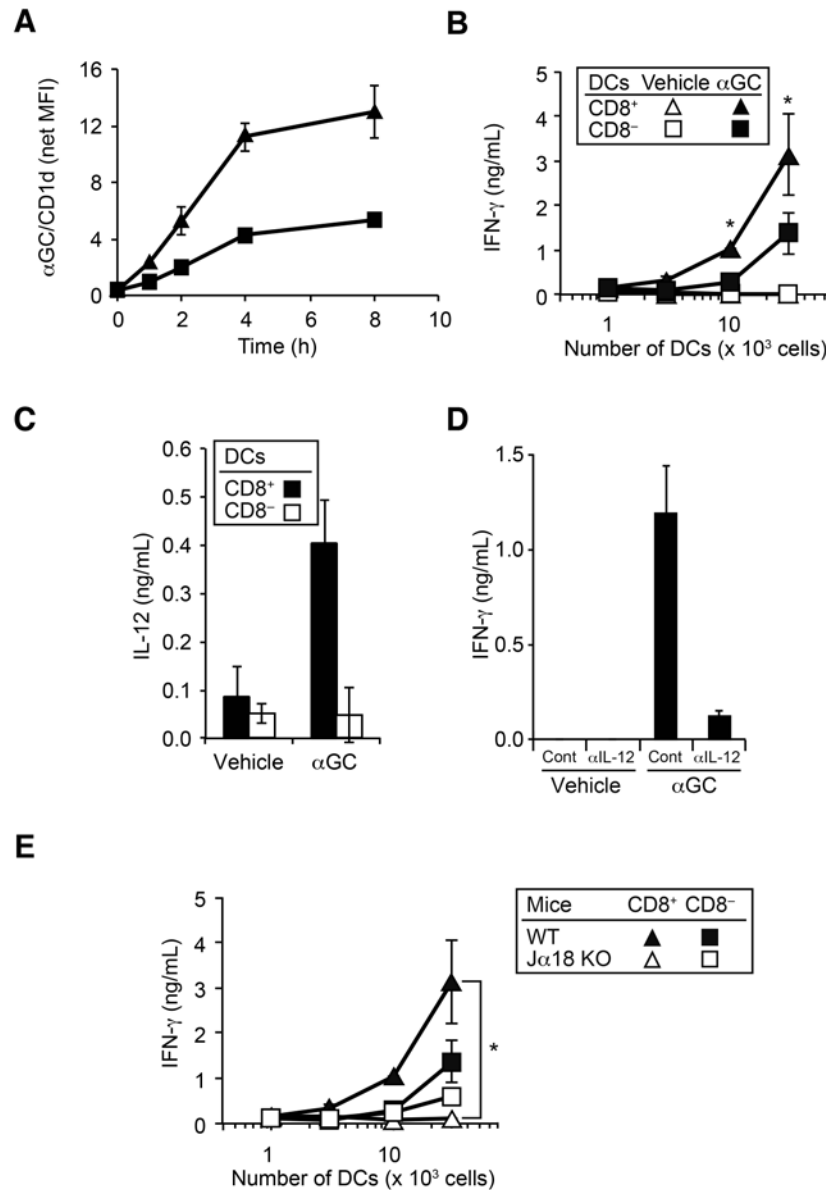
For iNKT cell preparation, splenocytes were depleted with anti-MHC II, CD8, CD62L, CD11b, CD16/32, and CD45R mAbs using Dynabeads, and dead cells were further removed with Dead Cell Removal kit (Miltenyi Biotec) in accordance with the manufacturer's protocol. The cells were stained with anti-CD19-FITC, α GC/Dimer X and anti-mouse IgG₁-PE, and then CD19⁻ α GC/Dimer X⁺ cells were sorted with FACS VantageSE.

For *in vivo* α GC loading experiments, cDCs were sorted after 2 h of *i.v.* injection of 5 μ g α GC. Various numbers of sorted cDCs were co-cultured with iNKT cells (1×10^4 cells/well) for 72 h, and IL-12p70, IL-4, and IFN- γ in culture supernatant were measured by ELISA. In the IL-12 neutralization assay, 10 μ g/mL anti-IL12 mAb or isotype control mAb was added in culture.

Reference

- [1] Iyoda T, Ushida M, Kimura Y, Minamino K, Hayuka A, Yokohata S, et al. Invariant NKT cell anergy is induced by a strong TCR-mediated signal plus co-stimulation, *Int. Immunol.* 22 (2010) 905-913.

2. Supplementary Figure



Supplementary Fig. 1. CD8⁺ DCs presented α GC/CD1d and induced IFN- γ production of iNKT cells more efficiently than CD8⁻ DCs.

(A) WT mice ($n = 3$) were injected *i.v.* with 5 μ g of α GC. At the times points indicated, α GC/CD1d complex expression on cDC subsets was measured by a flow cytometer. (B) Two hours after *i.v.* injection of 5 μ g of α GC, CD8⁺ DCs and CD8⁻ DCs were sorted and co-cultured with naïve spleen iNKT cells for 72 h. IFN- γ production was measured by ELISA. (C) IL-12 in supernatant culture as in (B) was measured. (D) IFN- γ in supernatant culture as in (B) with neutralizing anti-IL-12p40 mAb was measured. (E) Two hours after *i.v.* injection of 5 μ g of α GC in WT and J α 18 KO mice, splenic CD8⁺ and CD8⁻ DCs were co-cultured with iNKT cells for 72 h. IFN- γ production was measured by ELISA. * $p < 0.01$ by Student's *t*-test.